STUDIES ON CROSS-FERTILIZATION AND SELF-FERTILIZATION IN LYMNAEA STAGNALIS APPRESSA SAY [1]

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In 1817 Oken obtained fertile eggs from *Lymnaea auricularis* which were reared in isolation during their entire reproductive period. Baudelot (1863) reported both self-fertilization and cross-fertilization in *Lymnaea*. Pelseneer (1920) saw only one polar body extruded from the eggs of *Lymnaea* (three species), and concluded that reproduction in isolated snails was parthenogenetic. However, Colton, (1918) in *L. columella* and Crabb (1927a) in *L. stagnalis* observed two polar bodies and on the basis of their observations concluded that parthenogenesis did not occur. Colton further reported that, although self-fertilization did occur, cross-fertilization was the rule; Crabb reported that cross-fertilization was mechanically impossible (1927b). Seshaiya (1927) concluded from a study of breeding habits of *L. lutcola* that both cross- and self-fertilization occurred in this species.

Lang in 1900 claimed that self-fertilization could occur without self-copulation, while Künkel (1908) believed that self-copulation was indispensable to self-fertilization, basing his opinion in part on the observation of self-copulation in *L. auricularis* by Von Baer in 1835. Colton and Pennypacker (1934) reported that self-fertilization in *L. columella* for 93 generations did not decrease the viability of the strain. Boettger (1944), in his survey of the Basommatophora, concluded that self- and cross-fertilization were both common in this order. DeWitt (1954) found the percentage of hatching less in self-fertilized eggs of *Physa gyrina* than in cross-fertilized eggs.

The first genetic proof that both self- and cross-fertilization occur in snails was supplied by Diver, Boycott and Garstang (1925) in a study of the inheritance of inverse symmetry in *L. peregra*. Further proof was obtained in the study of the inheritance of albinism in this snail (Boycott and Diver, 1927). Ikeda and Mura (1934), using shell color as a genetic marker, demonstrated that both self- and cross-fertilization occurred in the land snail, *Bradybaena similaris*.

Bretschneider (1948a, 1948b) investigated the mechanism of insemination and oviposition in *L. stagnalis*. He reported that he had seen sperm balls leaving the seminal vesicle and being swept up the female tract to the hermaphroditic duct, where he assumed fertilization occurred. As additional evidence he reported seeing a complete spermatozoon inside the cytoplasm of an egg still in the duct.

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Holm (1946), in micro-anatomical studies on the reproductive tract of *L. stagnalis,* found a "fertilization pocket" homologous to that found by Meisenheimer (1912) in *Helix pomatia,* but saw no eggs or spermatozoa in it. Perrot (1940) reported a similar structure in *Limax maximus.* Abdel-Malek (1954a, 1954b) saw ova in the corresponding pockets in *Helisoma trivolvis* and *Biomphalaria biossyi.*

The present study was undertaken to determine: (1) the mode of inheritance of albinism in *Lymnaea stagnalis;* (2) the relative frequency of self-fertilization as compared with cross-fertilization in this species; (3) the survival time of spermatozoa after transfer from one snail to another; and (4) the possible location of fertilization of the eggs in this snail.

Materials and Methods

The snails used in this study were obtained from strains that had been maintained in laboratory culture at the University of Wisconsin for over ten years. Culture methods were those of Noland and Carriker (1946).

Mode of Inheritance of Albinism

Although albinism has been found to be inherited as a simple Mendelian recessive in several other gastropods (Boycott and Diver, 1927, in *L. peregra;* Ikeda, 1937, in *Philomycus bilineatus*), it was necessary to verify this for *L. stagnalis* before albinism could be used as a genetic marker in this study.

Accordingly two snails, one from the albino culture and one from the pigmented culture, were isolated until each had deposited at least one egg mass. The offspring from the eggs of the pigmented snail were all pigmented and those from the albino snail were all albinos. The two parent snails were then paired for 42 days. During this time one copulation was observed with the pigmented snail serving as the male. Presumably other such copulations occurred when the snails were not under observation.

After 42 days the two snails were separated. The albino was kept in isolation culture, and its egg masses were collected. The offspring resulting from these egg masses were examined under a binocular microscope six days after hatching. By this time pigment had developed along the mantle collar. Any young not showing pigment were re-examined after another five or six days. Of 885 offspring grown from 28 egg masses laid by the albino snail, 43 were albinos, resulting presumably either from self-fertilization or from previous copulations with other albinos in the original stock culture. The 842 pigmented offspring of the albino parent were clearly the result of fertilization of the eggs of the albino by spermatozoa from its pigmented mate.

Six of these pigmented heterozygotes were selected and isolated before sexual maturity. Each was maintained in solitary culture to insure that only self-fertilization would occur. This self-fertilization is obviously the equivalent of crossing two F_1 heterozygotes. Five or more egg masses were saved from each snail, and the progeny therefrom were grown to the age of "pigment-testing." Of a total of 4909 eggs (49 egg masses) from the six heterozygous snails, 64.7% hatched, and of those that hatched 92.9% survived to be examined for the presence of pigment. Of 2949 thus surviving, 2193 were pigmented and 765 were albinos. On a 3:1 basis the

expected ratio would have been 2212:737. The agreement is close and, on the basis of chi square tests, the difference between the expected and the observed ratio was not significant. It may therefore be concluded with confidence that albinism in *L. stagnalis* is inherited as a simple Mendelian recessive, as in other gastropods.

Prevalence of Self-fertilization

Eighteen albino snails (15 with pedigreed albino parentage and 3 from exclusively albino stock cultures) were paired, each with a homozygous pigmented snail taken from the stock culture which for ten years had shown no albinos. Each of these pairs was kept in a separate dish for varying lengths of time (from 20 to 187 days, depending on the pair). The albino partners were thereafter maintained in isolation culture. The eggs produced by these albinos were saved until the hatching snails reached the "pigment-testing" age to determine the relative numbers of pigmented and albino progeny. It was assumed that the albino progeny resulted from self-fertilization and the pigmented offspring from cross-fertilization. There was a slight possibility that the three snails, taken as adults from albino cultures, might have cross-copulated with other albinos before isolation. Two of these three snails showed 100% pigmented offspring in their first egg mass. The third was never seen to copulate with its pigmented partner, and produced only albino offspring throughout its life. Since all other snails were young (less than 130 days) and since no copulations had been observed in the cultures from which they were taken, the possibility that they had already cross-copulated with other albinos is extremely small.

Of the 18 albino snails paired with pigmented mates, 15 of them after separation produced mainly pigmented offspring during the first month, while three gave only albino progeny. From this it is clear that, when albino and pigmented snails are paired, not only does cross-fertilization occur, but, contrary to the opinion of Crabb (1927b), it is the predominating process.

Ten of the 15 albino snails that produced pigmented offspring after separation from their pigmented mate gave 100% pigmented young in at least one of their egg masses. In five of these it was the first egg mass laid after isolation that gave only pigmented progeny. One showed only albinos in its first egg mass, but by its third egg mass was producing 100% pigmented young. Of the ten snails that gave 100% pigmented offspring in at least one egg mass, four were producing albinos exclusively by the end of their lives. Three others, however, were still producing 100% pigmented progeny in the last egg mass laid before they died. Noland and Carriker (1946) have shown that snails maintained in solitary culture their entire lives frequently will produce more fertile eggs than snails allowed to cross-copulate. It is therefore unlikely that the continued production of pigmented offspring by the three snails mentioned above could have been due to any lack of fertilizing ability on the part of the animal's own sperm.

While cytological tests were not made to eliminate the possibility of parthenogenesis in the case of isolated snails, this seems unlikely because of the almost exact 3:1 ratio obtained in the offspring of the isolated heterozygous snails mentioned earlier in this paper. Had haploid parthenogenesis occurred, a ratio nearer to 1:1 would have been expected. If diploid parthenogenesis had occurred exclusively, only pigmented offspring would have been expected. Moreover, the work of Col-

ton (1918) and Crabb (1927b, 1928) indicated that two polar bodies are extruded by the eggs in *Lymnaea*.

Each of the 18 albino snails mentioned above was kept in isolation culture until its death, with one exception. This snail was discarded after producing nothing but albino offspring in its first five egg masses. The ages of the snails at death, in the 14 cases where it was known exactly, varied from 128 to 465 days. The latter figure represents the oldest snail ever reared in this laboratory.

Longevity of Transferred Sperm

The time elapsing between the separation of an albino snail from its pigmented mate and the laying of its last "pigment-producing" egg gives an approximate figure for the survival time of transferred sperm in the recipient snail. The maximum time found in this study was 116 days. To get some idea about how fast the fertilizing power of transferred sperm is lost, the data obtained from 13 of the 18 snails referred to earlier were combined. Of the five snails not used in the calculations, three (as mentioned above) had not received sperm from their pigmented mate, and two others died too early to give significant data.

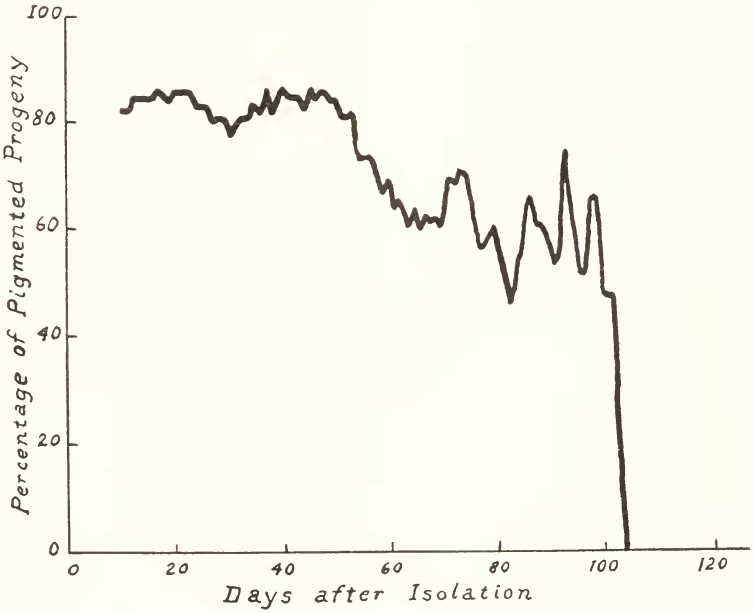

Figure 1. Graph showing rate of decrease in percentage of pigmented individuals in the offspring of albino snails that were isolated after receiving sperm from pigmented snails. (Data obtained from egg masses deposited by the albino after the 102nd day of isolation could not be treated in this graph.)

The data from the 13 remaining snails represented 260 egg masses, containing 15,545 eggs. Of these 72.5% hatched; and of those that hatched, 87.8% survived to the "pigment-producing" age. Figure 1 presents a curve showing the percentage of pigmented individuals in the progeny of the 13 snails plotted against time elapsed since separation from their pigmented mates. The curve represents a moving average, smoothed as follows: each point on the curve represents the total pigmented offspring developing from eggs laid in the 10 days just preceding a chosen point, divided by the total offspring produced from all the eggs laid during the same period. The curve thus represents substantially the percentage of cross-fertilization on successive days following separation.

Examination of the curve shows that in the first 50 days cross-fertilization was very high (over 80%), after which time it gradually fell, dropping rather suddenly near the 100th day. As stated earlier the maximum figure obtained was 116 days. Whether this figure really represents the maximum survival period of the sperm or merely the time at which the supply of transferred sperm was all used up, it is impossible to say.

To arrive at a more exact figure for sperm survival after transfer, it would be necessary (1) to add to the figure obtained (116 days) the time elapsing between the last copulation and the separation of the two partners, and (2) to subtract from the figure the time elapsing between actual fertilization and the laying of the egg. These corrections cannot be made from the data here obtained.

Location of Fertilization

The observations of Meisenheimer (1912), Holm (1946) and Abdel-Malek (1954a, 1954b) suggest that the sperm probably enters the egg in or near the "fertilization pocket." Bretschneider (1948a), however, thinks that fertilization may occur as high up in the reproductive tract as the hermaphroditic duct. (The anatomy of the reproductive system of *Lymnaea stagnalis* is shown in Figure 2.)

If foreign sperm after copulation actually travel up the female tract as far as the hermaphroditic duct, as Bretschneider implies, it would seem likely that they would mix with the sperm of the recipient snail. Then if such a mixture of sperm were later transferred in copulation, it is conceivable that some of the foreign sperm might be passed along to a third snail. This possibility was tested as described below.

Ten albino snails that had never been with pigmented snails were paired with pigmented mates until the albinos were seen to function as females in copulation with those mates. Each of these albinos was then marked with finger nail polish on the tip of the shell and placed with another albino which had never been with a pigmented snail. The pairs were maintained until the marked albino was observed functioning as a male in copulation with the second albino. Eggs were saved from the second albino after isolation, and young snails grown from them. In no case were any pigmented offspring obtained.

This negative result indicates either (1) that the transferred sperm did not reach the level of the hermaphroditic duct in any significant number, or (2) that foreign sperm cannot survive a second passage through the reproductive tract in the process of copulation and later movement up the female tract. Since the foreign sperm had already made such a passage once, it seems a bit unlikely that they could

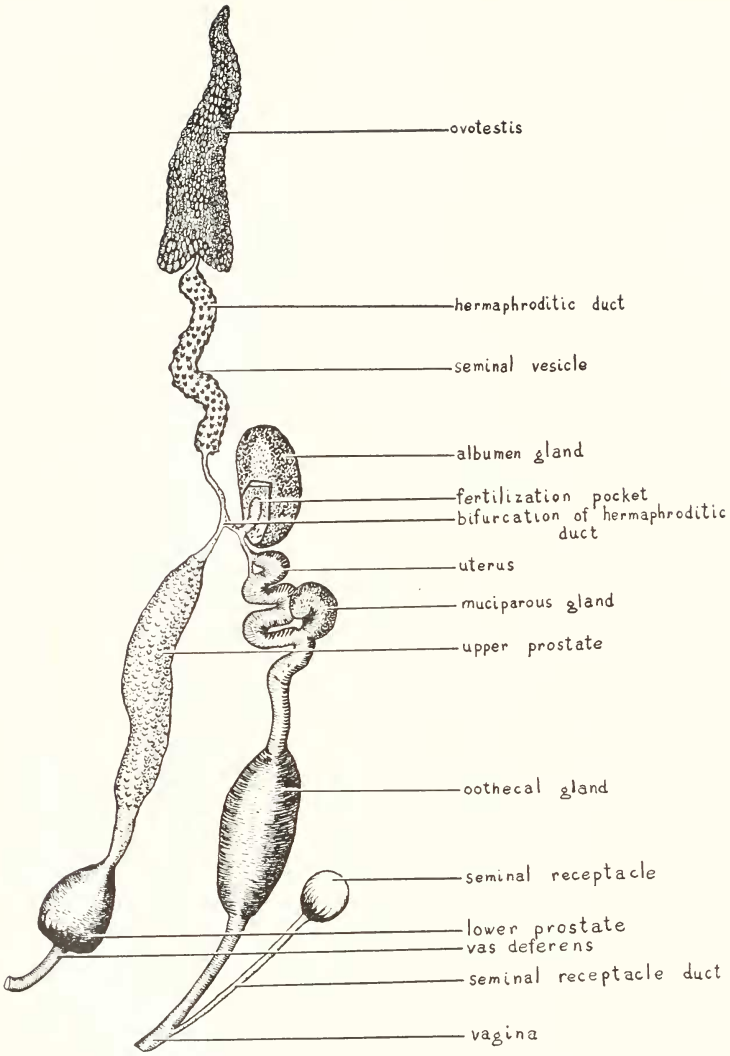

FIGURE 2. Dorsal view of the reproductive system of *Lymnaea stagnalis appressa* (× 40). The vas deferens has been cut away just beyond the point where it joins with the lower prostate gland. The copulatory apparatus is not shown.

not do it again without injury, though they would undoubtedly be greatly diluted by the other sperm with which they were transferred. These results therefore suggest that foreign sperm probably do not travel up the female tract as far as the hermaphroditic duct.

If, as this suggests, fertilization occurs below the bifurcation of the hermaphroditic duct in the oviduct, it must occur very high up in this latter structure, since the albumen and egg shell is laid down around the egg very soon after the egg enters the oviduct (according to Holm and Bretschneider), and no micropyle has ever been found in the snail's egg shell.

If fertilization does not occur above the point of bifurcation of the hermaphroditic duct, self-fertilization could result only after the transfer of sperm by self-copulation. That self-copulation actually does occur has been observed by many workers. Experiments were made to test this possibility.

Even though snails are extremely difficult subjects for surgical experimentation, the intromittent organ was successfully removed in 9 out of 14 cases. These snails continued to lay eggs after self-copulation was no longer possible. The obvious possibility of prior self-copulation could not be excluded. In several snails in which a section of the vas deferens was experimentally removed without subsequent death of the snail, regeneration re-established a connection. The question, therefore, remains unsettled as to whether prevention of self-copulation will also prevent self-fertilization.

The possibility that the seminal receptacle might serve as an activating organ for the sperm was excluded by examination of seminal receptacles removed from snails at different intervals following copulation. Only in those removed within 30 minutes after copulation were motile sperm found, and the motility was less than that of sperm taken from the vas deferens or ovotestis. The problems of the location of fertilization and the function of the seminal receptacle still remain unsolved.

Summary

1. Albinism in *Lymnaea stagnalis appressa* Say is inherited as a simple Mendelian recessive.

2. Cross-fertilization greatly exceeds self-fertilization in snails allowed to cross-copulate.

3. Transferred sperm may remain viable in the body of the recipient snail for as long as 116 days.

4. It is unlikely that foreign sperm are stored as high up in the reproductive tract as the seminal vesicles, since albino snails previously impregnated by pigmented snails and later mated to virgin albinos engender no pigmented offspring in the latter.

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